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Research Article

Effects of Alkaloid Rich Extract of *Citrullus colocynthis* Fruits on Artemia Salina and Human Cancerous (MCF-7 AND HEPG-2) Cells

Aniruddha Mukherjee*, Savita D Patil

R.C. Patel Institute of Pharmaceutical Education and Research, Shirpur, Dhulia, Maharastra -425405, India

***Address for Correspondence:** E-mail: savitad_patil@rediffmail.com

Abstract

In the present research the anti-cancer effect of alkaloid rich extract of *Citrullus colocynthis* fruits was investigated. The cytotoxic effect were evaluated in two phase, initially the effect of the extract were demonstrated on brine shrimp lethality assay (on freshly hatched napolii of Artemia salina). The effect of the extract exhibited strong cytotoxic effect (LC50=3.30μg/mL) due to its potent cytotoxicity components. On the second stage its anti cancer activity has been analysed on human cancerous cells. Initially the effect has been evaluated on MCF-7 cells which exhibited significant reduction in cell viability in dose dependant manner (LC50=17.2μg/mL) in very low concentration at 5μg/mL, 10μg/mL, 20μg/mL. Similar effect has been observed in human hepatoma cells (HepG-2), proved to be potent anti-cancer moiety (LC50=12.54μg/mL). These effects were observed maintained even after 48 and 72 hours (time dependant manner).

Keywords: Anti-cancer, Artemia salina, *Citrullus colocynthis (L)*

Introduction

Cancer has become one of the ten leading causes of death in India. At present there are 2.5 million cancer cases and nearly 0.8 million new cases occurring every year and 0.4 million deaths occurring every year.[1] However, the majority of cases, especially those that result in metastasis, are still treated with conventional chemotherapy. The problem of drug resistance is a major obstacle in chemotherapeutic treatment, therefore, there is a great need for the development of new therapeutic drugs that will be more efficient or will synergize with the existing ones. There has been a growing interest in the use of herbs as a potent source of new therapeutic anticancer drugs.[2] Plants contain a wide variety of chemicals that have potent biological effects, including anticancer act. Therefore, it is crucial to evaluate the potential of herbal remedies for the discovery of novel bioactive compounds that might serve as leads for the development of potent drugs.[3]

Citrullus colocynthis (L.) Schrad is a member of the family Cucurbitaciae. This plant, commonly known as the bitter apple or bitter cucumber, grows widely in the Arabian countries mainly in Sahara deserts, Sudan and India. The dried pulp of its fruit was used as a traditional medicine mostly for congestion, colic, constipation, dropsy, fever, worms, sciatica, constipation, laxative, scorpion bite etc.[4] Many workers suggested that the fruits of colocynthis possess antitumor activity, antibacterial and anticandidal.[5] The leaf showed laravacidal activity,[6] antidiabetic activity in human[7] as well.

There are various chemical constituents among which cucurbitcine glycosides were proved to be potent anti-cancer agent[8] but again its toxicity profile[9-10] kept the drug in the area of disinterest. Alkaloids are the secondary metabolites that presume to keep secure the plant by various insects; herbivores animals, keep these phytochemicals in the limelight of its probable cytotoxic activity.[11]

The present study has been planned to investigate the cytotoxic and anti-cancer effect of alkaloidal rich fraction isolated from the rind of the fruits of *C. colocynthis.*

Materials and methods

Materials

The fruits of the plant were collected from a shop (lokhande house) Pune, Maharastra, in the month of July. The samples were cleaned properly, air dried and finely powdered. The plant was authenticated by BSI (Botanical survey of India) BSI/WC/tech/2009-822-lot-4 Pune, Maharastra, India.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), hank's balanced salt solution, RPMI-1640, trypsin, DMSO were purchased from Hi-Media, Pune.

Extraction

The fruits were broken dried and size reduced to coarse powder in homogenizer. One kilogram the air-dried samples under investigation were defatted with light petroleum ether (b.p. 40º-

60ºC) in a continuous extraction apparatus. The marc was then dried, and mixed with calcium oxide and subjected to exhaustive percolation with 70% alcohol. The alcohol was distilled off under reduced pressure at 50ºC. A dark reddish-brown residue was obtained.[12]

Purification of the alcoholic residues

The alcoholic residue of the fruits under investigation was dissolved in 25 ml of alcohol 50%, the resultant solution was treated with 5 ml of lead' acetate (T.S.) and then filtered. The filtrate was separately treated with H_2S gas and filtered again. The excess lead was removed as sulphide by passing it through precipitated lead sulphide. The resultant filtrate was refluxed with few grams of animal charcoal for 3 hours and filtered. The filtrate was separately concentrated under vacuum to a small volume and left in an ice-chest for 24h; no crystalline substances were observed. The solution, in each case, was divided into 2 portions: one portion, was saved for the and TLC investigation, while the second was diluted with water (50 ml), rendered alkaline with sodium hydroxide and extracted several times with small portions of chloroform, until the chloroformic extract "failed to give" a color with Mayer's reagent. The combined chloroformic extracts were washed with water (100ml) and filtered over anhydrous calcium chloride. The chloroformic filtrate was distilled off and the residues obtained separately weighed.

Cytotoxic activity

Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of extracts of *Citrullus colocynthis*. Brine shrimps (Artemia salina) were hatched using brine shrimp eggs in a conical shaped vessel (1L), filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH under constant aeration for 48 h). After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in each vial containing 4.5 ml of brine solution. In each experiment, 0.5 ml of the plant extract was added to 4.5 ml of brine solution and maintained at room temperature for 24 h under the light and surviving larvae were counted. The same was followed using the control (vehicle treated) in different concentrations (1-5000 μg/ml) of the alkaloid rich fraction in a set of three tubes per dose.[13-14]

Lethality concentration determination

The percentage lethality was determined by comparing the mean surviving larvae of the alkaloid rich fraction and reference as podophyllotoxin. IC50 values were obtained from the probit method line plotted probit verses log dose.

Anti cancer activity

Cell line and culture

Human hepatoma cell line (HepG2) and human breast cancer cell line MCF-7 was obtained from the National Cell Culture Science (NCCS) Pune. Cells were cultured in complete Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in 5% carbon dioxide. Subcultures were performed by using 0.05% trypsin solution.

MTT assay

Exponentially growing cells were harvested, counted, and inoculated (at the appropriate concentrations in a volume of 100 μl) into 96-well micro titer plates; 3 replicates were prepared for each dose. Flat-bottom micro plates were used for MCF-7 and HepG-2 cells. Immediately after counting adds 10 μl of different concentration of alkaloid fraction of C. colocynthis (5, 10, 20, 40μg/ml) were added to each well. After different incubation times 24, 48, 72h at 37°C in a humidified 5% CO_2 atmosphere, the MTT assay was performed. MTT (Hi-Media, Pune) was dissolved at a concentration of 5 mg/ml in Hank's salt solution.[15-16]

Ten micro liters of MTT solution was added to each wells including the control wells without the cells. Additional controls consisted of only media without any cells, with or without the various drugs. After 4-6 h of incubation, micro titer plates were centrifuged at 2000 rpm for 10 min; medium was then removed, and 100 μl of DMSO was added to each well.[17] After thorough mixing with a mechanical plate mixer, absorbance of the wells was analyzed in a scanning well micro culture plate reader at wavelengths of 570 nm.

Statistical analysis

IC50 was obtained by best-fit line method line plotted against % cell viability verses log dose.

Results

Cytotoxic activity of alkaloid rich fraction of fruits of C. colocynthis in Artemia salina

Brine shrimp (Artemia salina) nauplii have been used in ecotoxicological studies since this assay responds to a broad range of chemical compounds and also effectively predicts pesticidal activities .The alkaloid rich fraction of fruits of C. colocynthis demonstrated strong cytotoxicity towards Artemia salina nauplii. Cytotoxicity (IC50 = 3.30μg/ml (Fig.1).

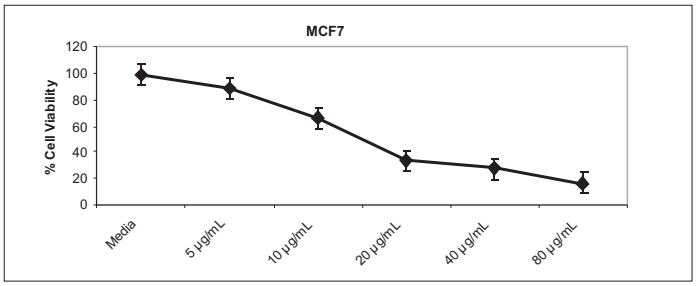

Fig.1: Anti cancer activity of alkaloid rich fraction of fruits *C. colocynthis* in MCF-7 cells.

The alkaloid rich fraction of fruits of *C. colocynthis* showed significant reduction in % cell viability among the MCF-7 cells in dose dependant manner at very low concentration (LC50 = 17.2 μg/ml). LC50 values were determined from best-fit analysis of three replicates. Comparison of cellular concentration of control and alkaloid rich fraction of fruits of *C. colocynthis* extract in 20μg/ml treated MCF-7 cells after 48h (Fig. 2a-b).

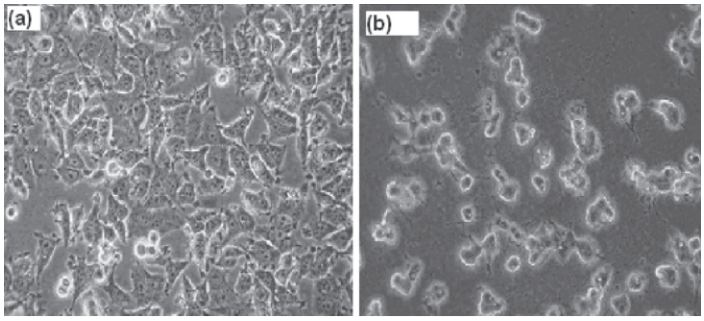

Fig. 2: Comparison of cellular concentration of control and alkaloid rich fraction of fruits of *C. colocynthis* extract in 20μg/mL treated MCF-7 cells after 48h. Key: (a) Control; (b) 20μg/mL of alkaloid rich fraction of *C. colocynthis* fruits after 48h.

Anti cancer activity of alkaloid rich fraction of fruits of *C. colocynthis* in HepG-2 cells

The alkaloid rich fraction of fruits of *C. colocynthis* showed significant reduction in % cell viability among the HepG-2 cells in dose dependant manner at very low concentration (LC50 = 12.54μg/ml). LC50 values were determined from best-fit analysis of three replicate (Fig. 3).

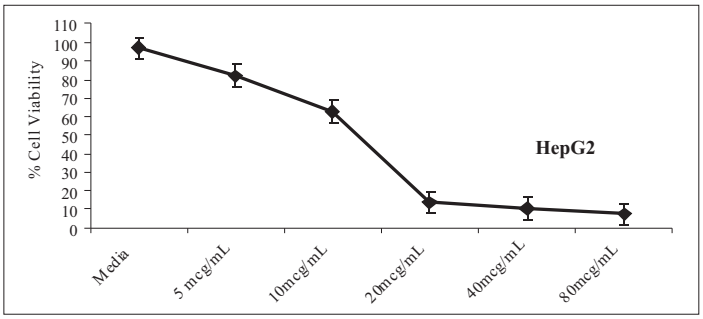

Fig.3: Anti cancer activity of alkaloid rich fraction of fruits of *C. colocynthis* in HepG-2 cells

Comparison of cellular concentration of control and alkaloid rich fraction of fruits of *C. colocynthis* in 20μg/ml treated HepG-2 cells after 48h (Fig. 4a-b).

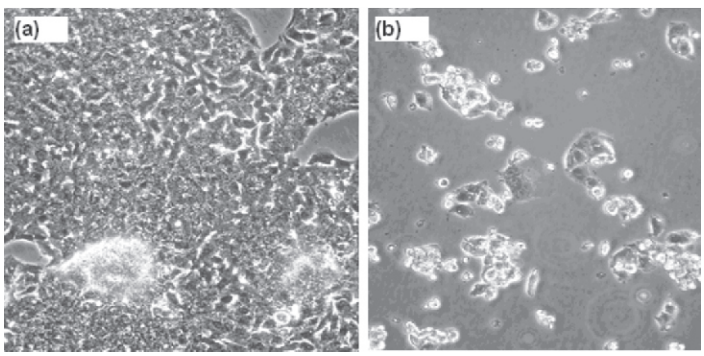

Fig 4: Comparison of cellular concentration of control and alkaloid rich fraction of fruits of *C. colocynthis* in 20μg/mL treated HepG-2 cells after 48 h. Key: (a) control; (b) 20μg/mL of alkaloid rich fraction of *C. colocynthis* fruits after 48 h.

The time dependant effect of extract on MCF-7 cells proliferation. MCF-7 cells at 5 $\times$103 cells/well were cultured with different concentration of alkaloid rich extract of *C. colocynthis* (5, 10, 20 μg/ml) in RPMI-10% FCS is observed. Daily cell proliferation was determined by MTT assay (Fig. 5).

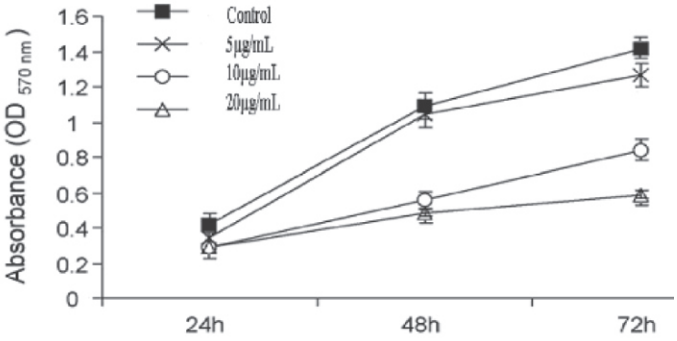

Fig. 5: Time dependant effect of extract on MCF-7 cells proliferation. MCF-7 cells at 5 $\times$ 103 cells/well were cultured with different concentration of alkaloid rich extract of *C. colocynthis* (5, 10, 20 μg/ml) in RPMI-10% FCS. Daily cell proliferation was determined by MTT assay.

The time dependant effect of extract on HepG-2 cells at 5$\times$103 cells/well concentration were cultured with different concentration of alkaloid rich fraction of fruits of *C. colocynthis* (5, 10, and 20 μg/ml) in RPMI-10% FCS was also observed. Daily cell proliferation was determined by MTT assay (Fig. 6).

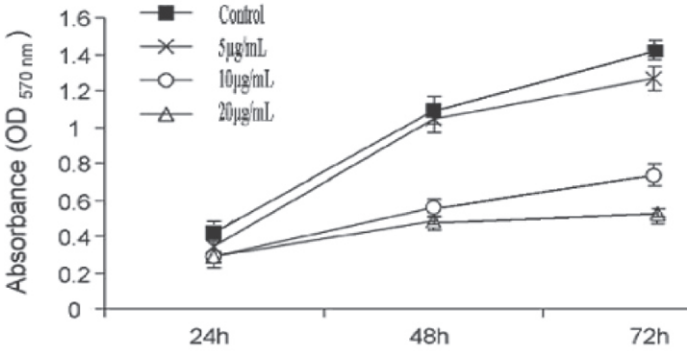

Fig. 6: Time dependant effect of extract on HepG-2 cells at 5$\times$103 cells/well concentration were cultured with different concentration of alkaloid rich fraction of fruits of *C. colocynthis* (5, 10, and 20 μg/ml) in RPMI-10% FCS. Daily cell proliferation was determined by MTT assay.

Discussion

In the present study, we studied the anticancer effect of alkaloid rich fraction isolated from *C. colocynthis* (L.) Schrad, fruits. In this study, the fruits of *C. colocynthis* were extracted with 70% ethanol,

treating with lead acetate and finally extracted with chloroform. Alkaloids were found in traces i.e. 0.02% could not be separated and it was further supported from literature.[18]

By various chemical tests, we found that principle chemical component was nitrogenous bases or alkaloids that come under pyridine or pyrimidine analogues.[12]

The alkaloid rich fraction of *C.colocynthis* fruits gave strongly cytotoxic activity against brine shrimp lethality assay (LD-50 3.30μg/ml). The result indicates that alkaloid rich fraction might posses some physiological activities, since the fraction was toxic to the brine shrimps. Furthermore, affirmed by the growth inhibitory activity of the fraction on human cancerous cells using MCF-7 and HepG-2 cells (Fig. 1 and Fig. 3), where the fraction rich with alkaloid exhibit significant growth inhibitory activity in a dose dependant manner as can be seen by MTT assay.

To evaluate the same effect we extended the experiment for further 72h, we found a prominent reduction in clusters of MCF-7 and HepG-2 cells that are quite prominent even after 48 h in both the cell population that once again reveals that the action is time dependant also (Fig. 5 and Fig. 6).

Significant reductions in number of clusters of the cells were found in 20μg/ml concentration (nearer to the IC-50 of MCF-7 cells 17.2 and IC50 in HepG-2 cells) where the clusters found were very less, in 40 μg /ml concentration in both the cell type (Fig. 2 and Fig. 4), increases the chances of the probability of anti adherent effect of the fraction on the human cancerous cells. On the other hand, the chemical nature of the fraction also revealed the probability of similar effects like various falls pyridine and pyrimidine bases.

Conclusion

The alkaloid rich fraction of *C. colocynthis* fruits for their cytotoxicity in Brine shrimp assay was observed and it was found that these extracts have strong cytotoxicity towards Artemia salina nauplii.

Considering the anti cancer activity in MCF-7 the alkaloid rich fraction of *C. colocynthis* fruits, has moderate reduction in cell viability in low concentration for long period of time. In hepatoma cells similar activity has been demonstrated and it was found that the alkaloid rich fraction has significant growth inhibitory action on human breast cancer and hepatoma cells.

In future, anti cancer activity of alkaloid fraction of *C. colocynthis* fruits can be further studied by separating its active component and its anti cancer activity on NCI 60 cell-lines, in-vivo models and nude or XCID animals. Further affirmation can be achieved by PK/PD (pharmacokinetic and pharmacodynamic) studies for its complete pre-clinical studies in terms of confirming the correct mechanism of action of the fraction.

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References

1. NCCP, Overview:National Canccer Control Programme, Pune, India. Available at: http.//www.who.int/cancer/modules/India.pdf. Accessed on 26.05.2012.

2. Denny, W, A., 2002. The Contribution of synthetic organic chemistry to anti-cancer drug development. Academic press 6th 187-202.

3. Shoeb, M., 2006. Anticancer agents from medicinal plants. Bangladesh Journal Pharmacology. 1, 35-41.

4. Decker, W., Deena S., 1999. Cucurbits～ Sanskrit～ and the indo-aryas. Economic" Botany, the New York Botanical Garden Press, Bronx, NY .53(1), 98-112.

5. Marzouka, B., Zohra, M., Rachel, D., Hayet, E., Halouid, E., Feninad, N., Aounia, M., 2009. Antibacterial and anticandidal screening of Tunisian Citrullus colocynthis Schrad. Journal of Ethnopharmacology, Short communication.

6. Abdul-Rahuman, A., Venkatesan, P., 2008. Larvicidal efficacy of five cucurbitaceous plant leaf extracts against mosquito species. Parasitology Rresearch.103, 133–139.

7. Huseini, H., F., Darvishzadeh, F., Heshmat, R., Jafariazar, Z., Raza, M., Larijani, B., 2009. The Clinical Investigation of Citrullus colocynthis (L.) Schrad Fruit in Treatment of Type II Diabetic Patients: A Randomized, Double Blind, Placebo controlled Clinical Trial. Phytotherapy research. Early view

8. Grossman, S., Bergman, M., Dovrat, S., Gottlieb, B., Hugo, E., Tannin-Spitz, T., 2007. A Growth inhibitory activity of cucurbitacin glucosides isolated from Citrullus colocynthis on human breast cancer cells. Biochemical pharmacology. 73, 5 6-67.

9. Adam, S., E., I., Al-Yahyaa, M., M., AL-Farhanb, A., H., 2000. Preliminary toxicity study on the individual and combined effects of Citrullus colocynthis and Nerium oleander in rats, Fitoterapia. 71, 385-391.

10. Adam, S., E., I., Al-Qarawi, 2003. Effect of Combination of Capsicum frutescens and Citrullus colocynthis on Growth, Haematological and Pathophysiological Parameters of Rats. Phytotherapy. Res. 17, 92–95.

11. Tadeusz, A., 2007. Biological Significance of Alkaloids. Alkaloids - Secrets of Life. 141-180.

12. Afifi, M.,D., Sayed, M.,S., Balbaa, S.,L., 1973. Nitrogenous bases of different organs of Citrullus colocynthis. Planta Medica. 24 (3), 260–265.

13. Meyer, B., N., Ferigni, N., R., Putnam, J., E., Jacobsen, L., B., Nichols, D., E., McLaughlin, J., L., 1982. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med. 45, 31–34.

14. McLaughlin, J., Rogers, R.,R., Anderson, J., E., 1998. The use of biological assays to evaluate botanicals. Drug information journal. 32, 513-24.

15. Mossman, T., 1983. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays.

Journal of immunological Methods. 65, 55-63.

16. Mallikarjuna Rao, C., Manjula, S., N., Mallehhappa Noolvi, N., Vipan Parihar, K., Manohara Reddy, S., A., Vijay Raman, I., Andanappa, K., Gadad, Gurdial Singh,. Gopalan Kutty, N., 2009. Synthesis and antitumor activity of optically active thiourea and their 2-aminobenzothiazole derivatives: A novel class of anticancer agents. Eur Journal of Medicinal Chemistry. 44, 2923–2929.

17. Supina, R., 1995. Methods m Molecular Biology, Vol 43: In Vitro Toxicity Testing Protocols, Humana Press Inc, Totowa, 43. 16, 137-150.

18. Nadkarni, K., M., 1976. Indian metaria medica. Popular prakashan, Mumbai. vol-1, 335-37.